to analyze the sample during processing by optical microscopy allows real time correction of incubation and wash conditions so as to further optimize signal to noise. PRE assays may be conveniently employed with essentially any target substance and various binding partners in direct, sandwich, and other widely used test formats, some of which are described in more detail below. Substances tested for and conjugated to PREs include proteins, nucleic acid, ligands, receptors, antigens, sugars, lectins, enzymes, etc.

Example 7

PRP Assay of Goat-Antibiotin

The wells of a polystyrene multi-well dish were coated with biotinylated BSA. Regular BSA was added to block any remaining non-specific binding sites in the wells. Samples of goat-antibiotin antibodies ranging in concentration from 0.06 to 600 picograms (pg) were added to individual wells. A control sample having no goat-antibiotin antibodies was also assayed. PRPs bound to rabbit-antigoat antibodies were then added to each well and incubated. Unbound PRPs were washed from the wells, and bound PRPs in each well were observed with a darkfield optical microscope. Light sources in the field of view were analyzed according to the discrimination techniques described above, and the remaining scattering sites were individually counted in each well. The results of this test are shown in Fig. 9. The control sample had one count remaining after image processing, and is illustrated as the dark bar in Fig. 9. The number of counted PREs over the concentration range tested varied from 4 at 0.06 pg analyte, to over 1000 at 600 pg analyte.

Because it is advantageous to perform these assays with one or more populations of PREs having approximately uniform spectral characteristics, it is advantageous to form the PRE labels first under conditions which are conducive to forming such approximately uniform populations. As mentioned above, the binding of nucleation centers to binding sites, followed by metal enhancement and optical observation has been described, but this technique provides very little control over the spectral characteristics of the particles thus created. And even when these techniques have been performed, no effort to use the PRE scattering characteristics to discriminate background and make a highly sensitive assay has been made or proposed. Accordingly, PRE assays performed by first labeling target species with nucleation centers, and then metal enhancing them to form PREs, (rather than forming the PREs prior to binding) are still improved when light discrimination between PRE scatterers and background is performed. Furthermore, assays which use pre-formed optically observable sub-wavelength light emitters of any kind have not taken advantage of the technique of individually counting particles to create a sensitive assay. When spectral and spatial discrimination of background is performed, such counting can be useful for fluorescent or luminescent bead labels in addition to PRE labels. As will be

* 1-45 missing

discussed below, fluorescence can be enhanced by local plasmon resonance, and thus PRE enhanced fluorescent beads provide an additional sub-wavelength light emitting label useful for such assays.

It can also be appreciated that many variations of these types of assays may be performed with PRE labels. All of the various types of immunosorbent assays which are currently performed using fluorescent molecule labels may be performed with PREs instead. Sandwich and competition assays, for example, may be performed with PREs. In the first case, an entity such as an antibody having affinity for a target substance to be detected may be immobilized on the bottom of an assay well. A test sample including the target substance is added to the well, and the target substance binds to the first entity. A second entity, having affinity for a different portion of the target substance, may then be added to the well, wherein it binds to the target species. Finally, PREs bound to a third entity having affinity for the second entity are added to the well, which bind in turn to the second entity. After rinsing, it can be appreciated that PREs will only be bound to sites where the target substance has been previously bound. This test is very useful when the first and second entities mentioned above are antibodies having affinity to different epitopes on an antigen being assayed. The third entity, bound to the PREs, may then be an anti-species antibody, rather than being a specific binding partner of the target substance.

In a competition assay, a first entity may be immobilized in an assay well, and both PRE coupled second entities and target substances are added to the well, wherein the second entity and the target substance compete for binding to the first entity. When unbound PREs are separated, the number of PREs remaining in the well indicates the extent to which the target substance was able to occupy binding sites. In this type of assay, the PRE bound second entity may be the same as the target substance, or may be a different substance which also has an affinity for the first entity.

Those of skill in the art will recognize that PRE labels may be used to bind to a wide variety of molecular complexes in a wide variety of ways to produce a sensitive assay. As additional examples, the conjugate on the PRE label may be a specific binding partner of the analyte being tested for. It may be a specific binding partner of an immobilized analyte/antibody complex. As another alternative, PRE may bind to an immobilized antibody, but only if that immobilized antibody has previously bound an analyte molecule. Each of these various techniques may be especially suitable in a given assay, depending on the chemical nature of the analyte being tested for.

Furthermore, it will be appreciated that assays for multiple analytes can be performed simultaneously using populations of PREs having different spectral signatures. Populations of PREs different color or different polarization responses can be conjugated so as to recognize different target substances. When introduced into a matrix containing unknown concentrations of several different

analytes, all of the assays set forth herein could be performed on several target substances at once by separately counting the PREs associated with each distinctive spectral characteristic.

PRE probes can also be used to screen *in vitro* combinatorial libraries. In some conventional versions of this technique, a drug receptor is labeled with a fluorophore then mixed with beads, the collection of which constitutes the combinatorial library, and spread out on a slide. The presence of a fluorescent bead indicates receptor binding and the presence of a potential drug bound to the bead. In one embodiment of the invention, the fluorescent receptor is replaced with a PRE-labeled receptor which increases the sensitivity and photostability of the assay, thereby allowing for the possible production of the original combinatorial library on smaller beads and the ability to synthesize and screen larger chemical libraries.

The libraries may also be synthesized on microchips, where the presence of a PRE probe indicates receptor binding. Recent applications of combinatorial libraries for improved drug discovery may thus be enhanced by using PRE probes as a method of detection of potential candidates. Selectively attached PRE increase the resolution and sensitivity of bio-chip detection schemes.

In all of these assays, PRE calibration is conveniently performed using PREs of different spectral characteristics than are used to detect the target entities. In essence, the assays are calibrated by introducing a predetermined quantity of PREs having a selected spectral characteristic to create a control population of PREs which can be detected and measured in conjunction with the PREs used for the assay function. As one specific sandwich assay calibration example, red PREs may be conjugated to the target entity being tested for, and a known amount (but of course much lower than a saturating amount) of this PRE conjugated target entity is added to the well along with the sample, either sequentially or simultaneously. After rinsing away unbound conjugated red PREs, antibodies to the target entity are added. After rinsing unbound target entity antibodies, blue PREs (for example) which are conjugated to an anti-species antibody are added which bind to the antibody to the target entity. After rinsing, both red and blue PREs are counted, and the red PRE count provides a calibration count. In an alternative to this sandwich format, a direct binding assay calibration may also be utilized, wherein different immobilized antibodies are provided on the bottom of the sample well, and the calibration PREs are conjugated to a specific binding partner to one of the immobilized antibodies.

Assays with PREs can also be performed in cells. Conjugated PREs can be bound to both fixed or free sites in cells and their locations individually observed. Well known techniques exist for placement of particles into cells, including high pressure bursts which cause the particles to perforate the cell membrane and electro-perforation in which high voltage discharges are used for the acceleration process (the PRE is typically charged prior to the electro-perforation techniques). Apparatus for performing these techniques are available from BioRad Laboratories of Hercules, CA. PREs and PRE

conjugates may also be introduced into cells by conventional transfection techniques including electroporation. PREs can also be placed into cells directly by piercing a cell membrane with a micropipette, and directly injecting one or more PREs into the cell. In a preferred embodiment, the PRE is coated (i.e. latex) by well known methods to protect it from biochemical damage.

In some advantageous embodiments of PRE assays within living cells, two populations of differently conjugated PREs are inserted into one or more cells. The separate conjugates associated with each separate population may be selected to bind to a different epitope on a target substance being manufactured in the cell. After injection into the cell, presence of the target substance will be indicated by PRE pairing, which is detected using the techniques described above. Depending on the nature of the target substance, it may be desirable to have PREs with similar, or disparate spectral characteristics associated with each conjugate.

It is advantageous to prepare wells for use with PRE assays which are suitable for observation with darkfield microscopy. For the multi-well plates to include a substrate suitable for darkfield microscopy, the well bottoms are advantageously manufactured with particular emphasis on uniformity, smoothness, and cleanliness so as to hinder the formation of light scattering imperfections. Such care is currently not taken in the production of standard 96 well dishes. In addition, the outside surface under the wells should also be relatively clean and smooth, as the outside surface also provides a light scattering surface which can introduce undesired background signals. In some advantageous embodiments, the surfaces of the wells have less than approximately 100, or even less than approximately 10, light scattering imperfections therein. As an additional method of increasing signal to noise ratios in these assays, the location of imperfections in a well can be documented, and a scattering signal from those locations can be ignored when the assay is performed with that particular well.

Typically, the field of view of the optical microscopes used in these assays comprises all of or portions of the bottom of the well. Thus, when low levels of analyte are being detected, it can be important to ensure that a minimum amount of analyte stick to the walls of the well, rather than to the bottom. It is accordingly advantageous to include a blocking agent on the walls of the well during production. To make such a well, a dish may be inverted and placed on a solution including a blocking agent such as BSA. If the dish is pushed down into the solution, or some of the air trapped in the wells is removed by sucking it out with a pipette or capillary, the BSA solution can be made to contact the walls of the well without touching the bottom of the well. After this step, the desired antibody or other binding agent is immobilized on the bottom of the well, and then additional blocking agent may be added to block remaining nonspecific binding sites on the well bottom.

Assay methods according to the invention can also be automated, employing, for example, the method and apparatus of the invention described in Section III. Automated plate readers are currently used for conventional assay techniques, and the principles for a robotic PRE plate reader are in some ways similar. As with currently available plate readers, a robotic sample loader may or may not be provided. A robotic PRE assay plate reader would advantageously include sample wells and a microscope for observing all or portions of the bottom of the wells. In some embodiments, a very small objective lens, which may be approximately 2 mm in diameter, is lowered down into the well and close to the well bottom to obtain a high numerical aperture while imaging a portion of the well bottom. In these embodiments, the PREs may be illuminated from the bottom with total internal reflection off the bottom of a transparent well bottom. As the light is gathered by the objective, light emitting entities can be detected and discriminated from background using automated image analysis techniques as described above. Counting the remaining discriminated particle sources can also be automatically performed. In some embodiments, the field of view of the microscope may be a portion of the assay well bottom, and the reader may be configured to discriminate and count particles in several regions of the same assay well until a certain predetermined count is read. Only after this count is reached will the reader move to a different assay well. This technique will save time by moving quickly from well to well when a large signal is present, and will take the time required to obtain adequate count statistics when low numbers of bound PREs are present in the well. The reader may also be configured to perform additional levels of discrimination depending on the count received. For example, a first discrimination based on the spatial deviation from the expected point spread function may be performed for all fields of view, but an additional spectral deviation measurement will be made when a low count value is obtained. All of the thresholds for performing various levels of discrimination can be preprogrammed into the reader, again insuring that wells having low PRE counts are analyzed to maximize signal to noise ratios, while time is saved on wells having a large number of counts, where signal levels are already high.

It will also be appreciated that mercantile kits including ingredients for performing assays described herein may be created having novel combinations of ingredients. Advantageously, such kits may include a container of PREs having approximately equal spectral characteristics. The PREs may be conjugated to selected biological molecules such as antibodies or other types of specific binding partners for selected substances. They may be coupled to reactive groups for custom formation of conjugate at a later time. Washing and blocking solutions may also be provided. A second container of PREs may also be provided for calibration or multiple assays as described above.

B. Binding of two PRPs to closely spaced target sites

As discussed above, the spectral characteristics of light emitted by PREs is dependent on their proximity to other PREs. Changes in observed peaks in emitted frequencies, e.g., peak wavelength, spectral width at half intensity, the appearance of more than one peak, and changes in response to polarized light, etc., can all be observed as PREs approach and move away from one another. These features can even be used to determine the approximate distance between PREs, by measuring the extent of their interaction.

Agglutination and aggregation-dependent immunoassays are thus performed using PRE probes, and have the capability of single molecule detection. In one embodiment, two antibodies are each attached to a PRE probe having either the same or distinct spectral signatures. These antibodies bind to the same biomolecule of interest, but at non-competitive sites. The distance between the two binding sites will place the PRE probes in close proximity which are directly detected via narrow band illumination if the two PRE have separated plasmon resonance frequencies or if they have the same plasmon resonance frequency, by a unique spectral signature as a result of their interaction. For example, blood serum is added to a tube containing PRE probes which have been coated with antibodies specific for a particular serum component. After incubation, the sample is spread on a glass slide and the frequency of aggregated (i.e. close proximity) PRE probes is determined. This is compared to control slides on which the serum would either contain or not contain the molecule of interest. This technique has application to the multi-PRE labeling and consequent detection of peptides, nucleic acid oligomers or genes, as well as portions of or whole cells or viruses.

The measurement of binding constants between two entities is currently performed by several procedures. Macroscopic binding can be measured directly by, for example, isothermal titration calorimetry. Less direct methods include absorbance, fluorescence or changes in circular dichroism associated with complex formation. One problem associated with these methods is that a high concentration of material is required to observe a detectable change in signal, and at these high concentrations the sample may be essentially 100% complexed, thus preventing the measurement of a binding constant under these conditions. In a preferred embodiment, the two entities are labeled with PRE probes, equilibrium is reached, and the ratio of free to bound allows calculation of a binding constant.

The ability to detect when two PREs are adjacent is also important for assays of molecular association and dissociation. If two PREs are associated with suitable conjugate pairs and are mixed together, they will bind to form a pair or, if not restricted, higher complexes. As one example, PREs conjugated to oligonucleotides will form such pairs or complexes if the oligonucleotide sequences on different populations of PREs contain complementary sequences, or if the PRE bound oligonucleotide sequences are complementary to separate regions of a target oligonucleotide also present in the matrix.

C. Cleavage of a linkage between two PREs

In this embodiment, a PRE is linked to another PRE thorough a cleavable linker, e.g., a peptide, oligonucleotide, oligosaccharide or other chemically or enzymatically cleavable linker. The aim of the linked composition is to detect single chemical or enzyme cleavage events, on the basis of an observable spectral change resulting from linked PREs becoming individual, unlinked PREs, in accordance with the Part B embodiment.

More generally, linked pairs of PRPs, are distinguished and, if the binding is disrupted, by, for example, enzymatic degradation of a peptide linker between the PREs or denaturation, this is reflected by the changes in the paired or complex PRE spectra. Operation of an enzyme may be monitored by this technique by observing an increased rate of complex formation or disassociation in the presence of the enzyme. One advantageous application of these methods includes monitoring the operation of a signal transduction cascade in a cell. Conjugated PREs are selected such that the presence of a product of a signal transduction cascade either disassociates previously bound PREs or binds disassociated PREs. The initiation of the cascade can thus be observed with optical microscopy in a living cell by observing association or disassociation of PREs in the cell.

Each PRE can be coated in such a way to result in a high probability of bound pairs by coupling with a linker such as a peptide or DNA molecule. As discussed herein, when two PREs with the same PR peak frequency are very close to each other, frequency shifts, additional resonances and polarization effects occur. If one wishes to determine whether a specific enzyme is present in solution, a linker is used which is susceptible to degradation by that enzyme. For example, serine proteases can be assayed by using a peptide linker containing a protease recognition site therein. After proteolysis, the spectra of the bound PREs changes dramatically as the PREs separate. In some cases, the PREs may be spatially separated far enough apart when linked such that they do not interact appreciably and retain essentially independent scattering spectra both when linked and unlinked. In this case, pair formation and disassociation can still be observed and measured by evaluating PRE positions with a CCD detector, and observe pairs of PREs having relative motion which indicates that they are linked.

VI. Additional Compositions and Applications of PREs

A. Monitoring Local Dielectric Environment

When a PRE in air is surrounded with a medium having a dielectric constant different from that of air, scattering parameters may change relative to the scattering parameters characteristic of the particle in air. This effect is reversible if the dielectric medium is removed. Such parameters include, for example, a change in intensity or shift in wavelength of the resonant peak, changes in the PREs response to polarized light, and a change in the number of resonant peaks. Shifts in the resonant peak and intensity are observed following the addition of liquids of different indices of refraction surrounding

the PRE, and after they are removed by suitable washing steps, the PREs exhibit their prior characteristics. For many materials which exhibit plasmon resonance, raising the index of refraction of the surrounding medium will red shift the resonant peak to a longer wavelength.

The presence of specific substances of interest or other perturbations in a sample being tested may therefore be detected by noting the spectral response of PREs to substances which interact with the PREs. For example, a suitable sample can be prepared having PRE bound to a substrate. Selected molecules may be bound to the PRE surface. The optical scattering parameters (intensity, polarization dependence, angular dependence, wavelength dependence, etc.) of each such PRE are recorded. The sample is then treated with material which includes molecules of interest that selectively bind to the surface of the PRE in such a manner that after initial treatment and/or subsequent further treatments, the PRE scattering parameters have changed, confirming the local absorption of additional material or desorption of the additional or initial material, or other changes in the local dielectric environment. It can be appreciated that the initial PRE sample may be prepared as a test "library" or used to screen an "applied " library of proteins, antibodies, etc. These peak

(D) Shift in Fluorescence Spectrum

shifts and intensity changes can also be used to detect molecular perturbations such as association and dissociation due to changes in the PRE local dielectric environment.

Information concerning the properties of a subject matrix can also be obtained by observing the spectral dependence on the relative positions of a PRE and a nearby substrate such as a smooth Si surface. For example, having made a record of a PRE location and spectral signature in a given sample, one could add an enzyme or photolyze a bond, resulting in movement of the PRE from the substrate, thereby changing the PRE spatial and/or spectral signature. Indeed, if a pair of such PRE were bound together, and one moved while the other remained bound to the surface, the resulting spectral signatures would clearly indicate this event. Coatings on substrates can also be used to provide further flexibility in creating detection and analysis systems utilizing PREs. For example, a coating can be applied to a substrate which will bind a desired polypeptide or polynucleotide or a blocking coating can be applied which will block non-specific binding of the PRE conjugates. One suitable coating comprises, for example, one or more layers of dielectric materials which produce anti-reflection properties. The coating may also comprise one or more layers of dielectric material which will produce an enhanced radiation by the PRE of the light that enters the observing optical system. The coating can also be selected so as to displace the PRE a distance away from the basic substrate surface. A given polarization of the light scattered by the PRE will be inhibited or enhanced depending on the distance from a reflecting surface. For example, if a suitable spacer layer of SiO_2 is placed on the silicon, a nice point source image peaked in the center is observed as expected. If the SiO_2 layer is adjusted or another

dielectric substance is used, conditions can be found related to the PRE resonant wavelength and the dielectric thickness and material where there is destructive and constructive interference of the PRE due to superposition of the light reflected from the substrate (interface) and the top of the dielectric layer. By using silicon or conducting surfaces, a noticeably different spectral signature is obtained than if the PRE moves away from contact with that surface or if a dielectric layer intervenes.

B. Monitoring Motion

Three dimensional PRE motion may be directly visualized using two observational lenses at right angles to each other, each yielding a two axis motion in the plane perpendicular to their respective optical axis. This is particularly suited to motions that are small compared to the depth of field of the objective lens. If the motion to be observed has a component which is large compared to the depth of focus of the objective lens, only one lens is used for three dimensional motion, whereby the "depth" direction is determined via a feedback signal that keeps the PRE intensity in focus on the image plane. The other two dimensions are determined in the usual manner. PRE distance from the substrate surface can also be monitored in TIR illumination systems by measuring the intensity of the light scattered by the PRE as it changes position. As the excitation electric field drops off exponentially with distance from the reflecting interface, PRE intensity will decrease as it moves away from the substrate surface.

Because PRE probes are so bright and so small they can be used for real-time determination of velocity and relative motion. For example, PREs may be used to monitor dynamic cellular processes including motor proteins (i.e. kinesin), cell division, vesicle transport, etc. PRE probes are particularly useful for *in vivo* temporal experiments over a broad range of timescales because they do not photobleach. PREs or precursor gold nucleating centers are attached to lipids which become incorporated into cell membranes. Specific PRE conjugates are designed to bind to their pair on cell surface receptors associated with the cell membrane. This method allows monitoring of, for example, ion channel openings. PREs may also be used to monitor movement of actin and myosin within muscle cells. PREs bound to or coated with conjugates can be introduced into cells. The conjugate will then bind to its binding partner within the cytosol, nucleus or on various organelle membranes. Activation of cell receptors, for example, by a particular drug, can lead to morphological changes in cell structure. PREs within or on cells can thus be used as an optical assay system for drug discovery or receptor activation. Once bound, the PRE can be localized and its motion observed. PREs may also be used to assay macroscopic motion. For example, a blood cell may be labeled and observed in circulation. Alternatively, the flow of blood or other liquid may induce a corresponding motion of the PRE. PREs can also be introduced into cells by a Biolistic device (BioRad Inc, Richmond, CA) or by electroporation.

By labeling any entity of interest with a PRE, the motion of that entity may be monitored using the detection process described herein by incorporating a suitable real time data acquisition and analysis system. Such a system may determine motion in a three dimensional sense and, if the movement is confined to a plane, in a two dimensional sense. Not only is precise information available about the motion in a specific system of interest, but also observable are changes in molecular motion after drug treatment or other changes in the physical and chemical environment such as alterations in temperature, pH, illumination, electric or magnetic field strength, or a change in concentration of any compound of interest.

PREs can also be used to monitor physical motion of more macroscopic objects. For example, a single PRE placed on an insect feeler could be used to sense its motion which could be regular or in response to an external molecule. This is particularly useful in detecting molecular responses to smell and pheromones. PREs are also ideal tools for allowing analysis of mechanical motion on a microscopic or sub-microscopic scale. By binding PREs to the components of so called "nanobearings" or other micron sized machine parts, three dimensional motion can be visualized and analyzed on nanometer scales. In addition to the added expense of electron microscopes, motion is difficult to capture via electron microscopy as the electron microscope is a scanning device, and the field of view is therefore generated over time with sequential scans, rather than viewed in real time as is possible with optical microscopy. Furthermore, with electron microscopy, extensive sample preparation is required, in addition to an evacuated measurement area. These factors severely limit the potential application of electron microscopy to real time motion analysis.

C. Near-Field Effects

The applications of PREs discussed above have focused on the far-field observation of light scattered by the PREs. However, because PREs also generate intense, non-radiative short-range electric fields, they may be used to affect the physical, chemical, and spectroscopic properties of adjacent molecules in useful ways. The spectroscopic technique of Surface Enhanced Raman Scattering may be extended to include the specific enhancement of only those materials in the immediate vicinity of the enhancing PRE. For example, PREs may be conjugated to bind to a target having a known Raman signature. Successful binding can be detected by observing the surface enhanced Raman spectra of the target. They can also be useful for locally enhanced excitation and modified emission of nearby fluorophores. Surprisingly, PREs can produce enhanced emission from even high quantum efficiency fluorophores if the surface of the PRE is placed from approximately 1 to 5 nm away from the fluorophore. In contrast, it is generally thought that the presence of a metal quenches fluorophore emission of high quantum efficiency fluorophores. This fact can be used to create fluorescent labels having a much higher brightness or a changed lifetime, compared to when not so associated. A label

which includes a plasmon resonant conductive core (such as a silver particle of 40-100 nm diameter) and a non-conductive shell, made, for example, from latex, may be created, wherein the shell has fluorescent or Raman-active molecules embedded on or within it. Preferably, the peak of the plasmon resonance has a significant overlap with the efficient excitation band for the fluorophore or Raman active molecule. When the label is illuminated, the plasmon resonance excitation of the core will greatly enhance the observed fluorescence. In accordance with the above discussion, the thickness of the non-conductive shell is preferably less than or equal to approximately 5 nm in order to produce fluorescence enhancement. The plasmon resonant core, selected to resonate at a chosen wavelength, thus dramatically improves label performance over the fluorescent latex particles currently commercially available.

Ellipsoidal PRE responses can also be advantageously employed in conjunction with fluorescence spectroscopy. Because ellipsoidal particles simultaneously permit resonance excitation at two or three distinct frequencies, they are particularly effective for localized excitation of a selected fluorophore by one such plasmon resonance, and then simultaneously effective for effecting the radiation (i.e. emission) of the excited fluorophore at wavelengths corresponding to the other plasmon resonance.

Thus, targeted PREs can induce very localized spectroscopic effects, again improving the collection of information about submicroscopic systems. Similar to the case of fluorescent resonance energy transfer (FRET), clustering of PREs gives rise to new optical properties including localized and Photonic Band Gap modes, which can be used to advantage in making highly responsive PRE-based detectors of molecular binding events.

D. Metrology and Instrumentation

Excited PREs can produce localized heating, and an individual PRE can be used to write to a polycarbonate substrate. As individual, highly localized light sources, PREs can be useful in precision lithography, photochemistry, or for inducing light activated chemical reactions.

PREs can also be used as markers in conjunction with all other non-optical forms of very high resolution microscopy, including electron, atomic force, and scanning tunneling microscopy. In these applications, a macromolecule of interest, such as a segment of DNA, is marked with one or more optically observable PREs. Preferably, the high resolution microscope is also equipped with darkfield optical microscopy apparatus for optically observing the portions of the surface to be imaged with the non-optical microscope. The PRE's bound to the molecule can be optically observed, and the relevant portion of the high resolution microscope, such as the atomic force or scanning tunneling probe tip, can be immediately positioned at a location of interest on the molecule to be observed. This can increase the efficiency of the use of high resolution microscopes, saving excess scanning time normally used to locate the object to be imaged. Atomic force, scanning tunneling, or any other type of scanning high

resolution microscope can advantageously be constructed to incorporate darkfield microscopy systems in order to utilize this feature of PREs.

Industrial applications requiring high precision alignment or registration may also benefit from the use of PREs. One such application is the semiconductor manufacturing process, where lithographic masks must be precisely aligned with the semiconductor wafer being processed. Because PREs can be localized to a precision of a few nanometers or even less, a comparison of the position of one or more PREs on the semiconductor wafer with the position of one or more PREs on the lithographic mask can determine the location of the mask relative to the wafer at the nanometer level. As only relative positioning is important, either random or controlled PRE patterns on the mask and the wafer may be used.

Another application of the present invention takes advantage of the fact that PREs are essentially point sources of optical frequency light, having a diameter much less than the emission wavelength. Thus, they produce only the point-spread-function pattern characteristic of the instrument through which they are viewed, and not an image of their structure. This point spread function can be analyzed to detect imperfections in the optical system used to create it. As one example, an angular variation in the intensity of the circular fringes indicates a lens in the viewing system which has a circumferential asymmetry. Localizing the center of the Airy pattern at two or more emission wavelengths also evaluates a lens systems for chromatic aberrations.

The point source nature of PREs can also be used to test an optical system for its resolution. Using techniques described above for the placement of individual PREs at specific locations, a calibration set of PRE pairs can be created with varying distance between the PREs. It can then be determined how close two PREs must be before the central peaks of their respective point spread functions overlap to produce a single non-differentiated peak. To some extent, the same measurement can be performed by measuring the width of the peak of a single PRE in the focal plane with the lens system of interest.

PREs may also be used to profile the intensity distribution of focused light beams, thereby gathering information concerning the properties of lenses and other optical systems used to produce such beams. As illustrated in Figs. 10A and B, a focusing lens 100 produces a light beam 102 focused to a narrow beam in the lens focal plane. As is well known in the art, the beam is not focused to a point at the focal plane, but the intensity has an approximately Gaussian intensity as a function of distance away from the center of the focused beam. The details of the intensity as a function of position in the focal plane will depend on the characteristics of the optical system which produced the focused beam.

Referring now to Fig. 10A, a thin transparent plate 104 may be placed in the beam 102 at the focal plane. The transparent plate 104 includes a PRE mounted thereon. Preferably, of course, the

peak of the plasmon resonance response of the PRE is selected to approximately match the predominant frequency band of the incident light beam 102. It can be appreciated that the intensity of the light scattered by the PRE will depend on the intensity of the illumination. Accordingly, if the plate 104 or beam 102 is moved such that the PRE moves to different locations in the focal plane, the intensity as a function of position can be determined by collecting scattered light with a suitably placed objective lens of an observing microscope. As with other darkfield techniques described above, the objective of the observing microscope may be placed so that it collects light emitted by the PRE but does not collect light transmitted through or specularly reflected by the plate 104. This system may be used to test the characteristics of solid-immersion-lenses, lasers, and other optical systems.

E. Object Identification

Still another application of the present invention is the labeling and identification of paper or plastic items subject to forgery such as paper currency or credit cards, or identification badges. Either random or pre-defined patterns of PREs may be bonded to the surface of the item. In advantageous embodiments, the PREs are coated with a protective layer or film. Later observation of the proper PRE pattern on the item with microscopy techniques as described above can be used for authentication purposes. Such authentication can be implemented via a pattern recognition system on a computer, allowing for real time authentication at point-of-sale terminals, facility entry locations, and the like. Alternatively, a magnetic strip, bar code, or other data storage media may be placed on the item (e.g., a credit card) in addition to the PRE arrangement. A coded version of the PRE array is also stored in this media, and a match indicates that the item was validly created. Of course, a cryptographic algorithm which produces a matching magnetic code based on the PRE array that cannot feasibly be deduced from the array itself should be used, and such algorithms are well known in the cryptographic art.

G. Forensics

The robustness and easy visibility of PREs also makes them ideal for several forensic applications. Bodily oils, fluids, DNA, etc. which is present in fingerprints can bind PREs, making the fingerprint easily visible under appropriate illumination. Many different goods may also be labeled with PREs to provide traceability. PREs having different scattering characteristics can be mixed with explosives, food, drugs, poisons or other toxins, etc. The particular PRE could provide source identification. PREs are ideal for this application because of their resistance to degradation and the ability to detect even single individual PREs in a sample.

H. Identifying small molecules in combinatorial libraries by Raman spectrum PREs

PREs can also be differentiated by the characteristics of molecules which are attached to their surface which may be provided in addition to the one or more conjugate molecules. Surface enhanced

Raman scattering from Raman active molecules adjacent to individual PREs has been reported (Nie and Emory, *Science*, Volume 275, 1102-1106, 1997). If molecules with different Raman spectra are attached to different populations of PREs, PREs from different populations may be identified by their different Raman scattering signatures. Given the wide variety of Raman molecules available, a large number of differentiable probes are possible which may be particularly useful in conjunction with combinatorial library techniques. The use of Raman markers may also be used as an alternative way (in addition to four different plasmon resonance wavelengths) to produce four differentiable PRE populations, which would be useful in DNA sequencing techniques which use four differentiable markers, one for each base. Fluorescent molecules may also be bound to PREs to provide an additional marker, as can oligonucleotides, which are distinguished by their preferential hybridization properties rather than spectrally. If desired, PREs having a conductive resonant core and a non-conductive dielectric shell such as latex may include embedded fluorescent molecules in the dielectric shell. This label embodiment is discussed in more detail below. It can be appreciated as well that combinations of different resonant scattering characteristics, different fluorescent markers, and different Raman markers can be utilized to prepare hundreds or even thousands of spectrally differentiable probes. For example, a library may include four different plasmon resonance signatures, four different fluorescent signatures, and ten different Raman signatures, thereby producing 160 different distinguishable probes by different combinations of the available spectral signatures. Accordingly, populations of PREs may be distinguished based on differences in size or shape, or by differences in material bound thereto.

Furthermore, known techniques of combinatorial chemistry can be used to simultaneously synthesize a marker molecule and a conjugate molecule onto PREs in a simultaneous series of molecular assembly steps. In some embodiments, this process would start with a label precursor entity which comprises a PRE having one or more reactive groups bonded to it which may form a base on which combinatorial chemical synthesis may initiate. The reactive groups may include, for example, a phosphates, aldehydes, carboxyls, alcohols, amides, sulfides, amino acids, or nucleic acid bases. For example, a selected Raman active molecule could be synthesized simultaneously with an oligonucleotide conjugate. Alternatively, a library of drug candidate compounds may be synthesized simultaneously with identifying oligonucleotide markers.

I. Cell Sorting

PRE probes are also suitable for cell sorting, analogous to fluorescent activated cell sorting (FACS). A mixed cell population is analyzed for one cell type expressing a particular surface antigen using a particular PRE probe. In addition, several cell types are isolated by simultaneously using multiple PRE probes because of the number of uniquely identifiable PRE probes with distinct spectral signatures that can be made. It is contemplated that all of the PRE detection and localization methods

described herein can be fully automated to produce, among other items, cell sorters. With a PRE cell sorter, it is advantageous to pass the cell population of interest substantially one at a time into the field of view of a darkfield microscope. The detection, discrimination, and analysis techniques described in detail above can be used in the cell sorting context to identify PRE labelled cells.

Many different cell routing schemes may be used in such an apparatus. In one advantageous embodiment, the cells are deposited into a stream of fluid, such as water, which is constrained to move within the confines of a surrounding shell of a second fluid, such as an oil, which is substantially immiscible in the first fluid. This forces the cells to remain confined to a small region for darkfield viewing as they pass through the field of view of the microscope. Preferably, the indices of refraction of the two fluids are approximately equal, to minimize reflections of incident light at the interface between them.

In addition, PRE labeling can be used in addition to, rather than as a substitute for, fluorescent labeling in a cell sorting technique. In this case, fluorescent labels and PRE labels are made to bind to the same target cells. The cell sorting may be done based on an observation of the fluorescent marker. If a portion of the sorted cells are saved as an archival record of the result of the sorting process, the PRE can be used to verify successful sorting in the future. This is more effective than observing the fluorescence of stored samples, due to the stability and non-photobleaching properties of the PRE.

A further application of the same technology is performed *in vivo* or *ex vivo*. In this technique, cells are permeabilized and PRE probe(s) attached to antibodies against a cellular biomolecule of interest are introduced into the permeabilized cells. The cells are then incubated with a combinatorial chemical library. The viable cells are spread out on a slide and the cells are selected which have been "affected" by the chemical library. "Affected" could indicate a change in localization or distribution of PRE probes due to a change in localization of the attached biomolecule, or it could indicate a clustering of PRE probes leading to a new spectral signature. Because any entity of interest (i.e. cell, DNA, organelle) can be labeled with a PRE, it can then be optically detected because the collection of PRE can be observed moving as a unit.

J. Clinical Applications of PREs

PREs can also be used in a wide variety of clinical applications. One significant area is in the diagnosis of different conditions in animals, including humans, which can be identified by the selective binding of conjugate to specific organs in the animal. In this technique, PREs having selected scattering characteristics may be injected into the bloodstream or ingested by the animal. These PREs may further be bound to an antibody or other conjugate to target or identify the presence of a particular substance in the animal. Tissue may then be removed form the animal and tested for the presence of PREs under a microscope. If desired, control PREs which are not bound to the specific binding conjugate can also

be injected or ingested to determine the non-specific binding background. These techniques have been developed with colored latex particles as the probe, and reagents for performing these tests with the latex particles are commercially available from, for example, Triton Technologies of San Diego, CA and Molecular Probes of Eugene, OR. The use of PREs, due to their brightness, biocompatibility, and resistance to degradation will improve the sensitivity of such tests.

Cell modification and therapy techniques such as gene therapy may also be enhanced with PREs. In this case, cells having the desired genetic characteristics are labeled with PREs and selected with a cell sorter using the techniques set forth above. Selected cells are then placed in a patient. If desired, the PRE can be disassociated and removed prior to placement in the patient.

Selective heating and drug delivery is also possible with PREs. If PREs are localized in a selected tissue or region of a patient, they can be illuminated so as to locally heat the tissue or region without significant affect on neighboring areas of the body. The administration and activation of light activated drugs is also enhanced with PREs. Light activated drugs can be activated with far less total light energy by being bound to a PRE where the electric field will be enhanced. The use of light activated drugs to treat breast cancer has received recent attention, and may be improved by binding the drugs to PREs to enhance their activation at locations deeper in the tissue.

The application of optical PRE detection and analysis to biochemical systems is considered to provide many advantages over current labeling techniques, and appears to comprise an area where PRE analysis can have a large impact. Other areas, however, may also benefit from the PRE detection and spectral analysis of the present invention.

From the foregoing, it will be appreciated how various objects and features of the invention have been met. The method and apparatus of the invention are ideally suited to a variety of target-interrogation tasks that have been difficult or impossible heretofore, including, as representative examples:

1. detecting single molecule events;
2. resolving sub-wavelength distance relationships in a biological target in a natural hydrated state;
3. direct spatial mapping of selected target sites on a biological target, such as direct mapping of selected sequences in a chromosome for purposes of chromosome mapping; and
4. optical reading of microencoded information;

The method and apparatus can further be applied to a wide variety of diagnostics applications, to achieve improved sensitivity, spatial and distance information, ease of sample preparation, and flexibility in the type of target sample that can be interrogated.

Although the present invention has been described with respect to particular methods, compositions, and devices. It will be appreciated that various changes and modifications can b made without departing from the invention.

WHAT IS CLAIMED IS:

1. A method of interrogating a field having a plurality of PREs distributed therein, comprising illuminating the field with an optical light source,

detecting a spectral emission characteristic of individual PREs and other light scattering entities in the field,

constructing a computer image of the positions and values of the emission spectral characteristic of individual PREs and other light-scattering entities present in the field, and

discriminating PREs with a selected spectral signature from other light-scattering entities based on detected spectral characteristic values unique to the selected-signature PREs, to provide information about the field.

2. The method of claim 1, wherein said detecting includes simultaneously detecting the spectral emission characteristic of the light-scattering entities in the field.

3. The method of claim 2, wherein said detecting further includes detecting the spectral emission characteristic of the light scattering entities in the field simultaneously at a plurality of defined spectral frequencies.

4. The method of claim 1, wherein said illuminating and detecting steps include

illuminating said PREs with incident light predominantly in a first frequency band;

detecting the spectral emission characteristics of individual PREs and other light scattering entities in the field under illumination at the first frequency band;

illuminating said PREs with incident light predominantly in a second frequency band; and

detecting the spectral emission characteristics of individual PREs and other light scattering entities in the field under illumination at the second frequency band.

5. The method of claim 1, wherein said detecting includes sequentially detecting the spectral emission characteristic of individual PREs and other light scattering entities in the field at a plurality of defined spectral bands.

6. The method of claim 1, wherein said illuminating includes exposing the field to a plurality of narrowband pulses of light which vary in duration, and said detecting includes detecting variations

in emitted light intensity produced by variations in duration.

7. The method of claim 1, wherein at least some of the PREs are non spherical, said illuminating includes exposing the field to polarized light at different orientations and/or different angles of incident, and said discriminating includes detecting changes in a spectral emission characteristic as a function of incident light polarization orientation or angle.

8. The method of claim 1, wherein said PREs are formed in the field by a step selected from the group consisting of

(i) binding nucleation centers to a field, metal enhancing said nucleation centers, observing enhancement of said nucleation center during said metal enhancing process, and terminating enhancement when a PRE of a desired spectral characteristic has been formed;

(ii) adding pre-formed PREs to a target in the field,

(iii) making PREs at target sites in the field.

9. The method of claim 1, wherein discriminating PREs with a selected spectral signature from other light-scattering entities in the field includes discriminating a selected type of PRE from all other light-scattering entities in the field, based on detected values, for each light-scattering entity in the field, of peak position, peak intensity, or peak width at half intensity of the spectral emission curve, peak halfwidth in the image plane, and polarization or angle of incidence response.

10. The method of claim 9, wherein said discriminating is effective to discriminate, for a selected type of PREs, those selected PREs which are interacting with one another and those which are not.

11. The method of claim 9, wherein said discriminating is effective to discriminate a selected type of PRE from another selected type of PRE in the field.

12. The method of claim 1, wherein the PREs have surface-localized fluorescent molecules or Raman-active molecular entities, and said detecting includes detecting plasmon-resonance induced fluorescent emission or Raman spectroscopy emission from one or more of said molecules or entities, respectively.

13. The method of claim 1, for use in determining the total number of PREs of a selected type in a field, wherein said discriminating includes counting the number of PREs having a selected range

of values of a selected spectral emission characteristic in the constructed computer image.

14. The method of claim 1, for use in determining a spatial pattern of PREs having a selected range of values of a selected spectral characteristic in the field, wherein discriminating includes constructing an image of the relative locations of PREs with those spectral-characteristic values.

15. The method of claim 14, wherein the location between two adjacent PREs is less than the Rayleigh resolution distance, and said detecting includes exposing the field with light of one wavelength, to obtain a diffraction image of PREs in the field, exposing the field with light of a second wavelength to obtain a second diffraction image of PREs in the field, and comparing the distance between peaks in the two diffraction patterns.

16. The method of claim 1, for use in interrogating a change in the environment of the field, wherein said discriminating includes comparing the values of the detected spectral characteristic of a PRE in the field before and after said change.

17. The method of claim 16, wherein the field is interrogated for changes in the dielectric constant of environment.

18. The method of claim 1, for use in detecting motion of PREs in the field, wherein said detecting includes detecting the centers of the diffraction patterns of the PREs in the image plane, as a function of time.

19. Apparatus for use in the method of claim 1, for interrogating a field having a plurality of PREs distributed therein, comprising

an optical light source for illuminating the field,

an optical detector for detecting a spectral emission characteristics of individual PREs and other light scattering entities in the field, when the field is illuminated by the light source,

an image processor operatively connected to the detector for constructing, from signals received from the detector, a computer image of the positions and values of the spectral emission characteristic of individual PREs and such other light-scattering entities present in the field,

discriminator means for discriminating PREs with a selected spectral signature from other light-scattering entities in the computer image, and

output means for displaying information about the field based on the information about the selected PREs.

20. The apparatus of claim 19, wherein said light source includes a bright field/dark field lens for directing light onto the field.

21. The apparatus of claim 19, wherein said light source includes means for illuminating the field at each of a plurality of different wavelengths.

22. The apparatus of claim 19, wherein said detector is a two-dimensional photodetector array capable of detecting a spectral emission characteristic simultaneously from a plurality of illuminated PREs in an illuminated field.

23. The apparatus of claim 19, wherein said detector includes means for spectrally separating light emitted from the PREs, and said image processor operates to form a computer image of the positions and values of the emission spectral characteristic of individual PREs and such other light-scattering entities present in the field at each of a plurality of different emission wavelengths.

24. The apparatus of claim 23, wherein the optical detector includes a two-dimensional array of optical fibers whose output is aligned so as to constitute a line source that is sent into a grating or prism for responding to that line source, and a two-dimensional detector array for responding to the spread of spectral light of each fiber in said line source of detected light.

25. The apparatus of claim 19 or 23, which further includes means for moving said target in an x-y plane, relative to said light source, to successively illuminate individual light-scattering entities in the field.

26. The apparatus of claim 19, wherein said image processor operates to construct an image of PRE positions and, for each light-scattering entity in the field, values of a spectral characteristic selected from the group consisting of peak position, peak intensity, or peak width at half intensity of the spectral emission curve, peak halfwidth in the image plane, and polarization or angle of incidence response.

27. The apparatus of claim 19, wherein said image processor operates to construct an image of PRE positions and, for each light scattering entity in the field, a value of a spectral characteristic selected from the group consisting of fluorescence emission spectrum and Raman spectrum.

28. The apparatus of claim 19, wherein said discriminator means includes means for

discriminating PREs with a selected spectral signature from all other light-scattering entities in the field, based on detected values, for each light-scattering entity in the field, of peak position, peak intensity, or peak width at half intensity of the spectral emission curve, peak halfwidth in the image plane, and polarization or angle of incidence response.

29. The apparatus of claim 29, wherein said discriminating is effective to discriminate for a selected type of PREs, those selected PREs which are interacting with one another and those which are not, or one selected type of PRE from another selected type of PRE in the field.

30. A composition of plasmon resonant particles (PRPs) having one or more populations of PRPs, and characterized by:

(a) the PRPs have a width at halfheight of less than 100 nm;

(b) the PRPs in a single population are all within 40 nm of a defined wavelength;

(c) at least 80% of the PRPs in the composition satisfying criterion (a) are in one or more of the populations and have a spectral emission wavelength in a single range selected from the group consisting of:

(i) >700 nm;

(ii) 400-700 nm; and

(iii) <400 nm; and

(d) each population has at most a 30% overlap in number of PRPs with any other population in the composition.

31. The composition of claim 30, wherein at least 80% of the PRPs in the composition are in one or more of the populations and have a spectral emission wavelength in the 400-700 nm wavelength range.

32. The composition of claim 30 or 31, wherein the particles have a composition selected from the group consisting of

(i) a solid silver particle,

(ii) a silver particle with a gold core, and

(iii) a particle with a dielectric core and an outer silver shell of at least about 5nm.

33. The composition of claim 30, wherein the particles have localized at their surfaces, one from the following group: (i) surface-attached ligands adapted to bind to ligand-binding sites on a target, where the ligand/ligand-binding sites are conjugate binding pairs, (ii) fluorescent molecules, (iii) Raman-

active molecular entities, and (iv) a blocking reagent to prevent non-specific binding, and (v) a coating with functional groups for covalent coupling to the PRPs.

34. The composition of claim 33, wherein the surface localized ligand is one of a conjugate pair selected from the group of pairs consisting of antigen/antibody, hormone/receptor, drug/receptor, effector/receptor, enzyme/substrate, lipid/lipid binding agent and complementary nucleic acids strands.

35. The composition of claims 33, which includes first and second populations of PRPs having first and second different surface localized molecules or entities.

36. The composition of claim 35, for use in identifying a target having first and second ligand-binding sites, wherein the first and second surface bound molecules are first and second ligands effective to bind to said first and second ligand-binding sites, respectively.

37. The composition of claim 36, wherein the first and second surface-localized molecules are oligonucleotides having sequences that are complementary to first and second proximate sequence regions of a target polynucleotide.

38. The composition of claim 35, wherein the first and second surface-localized entities are Raman-active molecular entities with different Raman spectral characteristics.

39. The composition of claim 30, having first and second populations of PRPs, each with a different shape, at least one of which is spherical or tetrahedral.

40. A diagnostic method for use in detecting the presence of, or information about, a target having a molecular feature of interest, comprising

contacting the target with one or more PREs having surface localized molecules, to produce an interaction between the molecular feature and the localized molecules,

illuminating the target with an optical light source, and

determining the presence of or information about the target by detecting a plasmon resonance spectral emission characteristic of one or more PREs after such interaction with the target.

41. The method of claim 40, wherein said target contains a ligand-binding site, the surface-localized molecule is one of a ligand/ligand-binding site conjugate pair selected from the group of pairs consisting of antigen/antibody, hormone/receptor, drug/receptor, effector/receptor, enzyme/substrate,

lipid/lipid binding agent and complementary nucleic acids strands, said contacting produces a PRE/target complex, and said detecting includes detecting a plasmon resonance spectral emission characteristic of the complex.

42. The method of claim 41, wherein said contacting further includes the step of washing the field to remove PREs not bound to the target through a ligand/ligand-binding interaction.

43. The method of claim 41, wherein the target has at least two proximately spaced ligand-binding sites, and said complex includes at least two proximately spaced PREs that have a spectral emission signature different from that of PREs in the absence of binding to the target.

44. The method of claim 43, for determining the presence of a target having first and second proximately spaced ligand-binding sites, wherein said contacting includes reacting the target with first and second populations of PREs having surface-localized first and second ligands, respectively, for binding to the first and second ligand binding sites, respectively.

45. The method of claim 44, wherein the target is a polynucleotide having first and second adjacent base sequence regions, the ligand molecules on the first and second PREs are complementary to said first and second regions, and said contacting is carried out under conditions which allow surface-attached ligand molecules to hybridize with complementary-sequence regions of the target.

46. The method of claim 41, wherein the PRE(s) contain surface-localized fluorescent reporter molecules, and the interaction of a PRE with the target or with another PRE at the target is effective to detectably alter a plasmon-resonance induced spectral emission characteristic of the fluorescent molecules on the PRE.

47. The method of claim 41, wherein the PRE(s) contain surface-localized Raman reporter molecular entities, and the interaction of a PRE with the target or with another PRE at the target is effective to detectably alter a plasmon-resonance induced spectral emission characteristic of the Raman entities on the PRE.

48. The method of claim 41, wherein the target has multiple ligand-binding sites, the PREs bind to two or more of these sites and said detecting includes constructing a spatial map of the sites of PRE attachment to the target, which is indicative of the relative spacings of the ligand-binding sites in the target.

49. The method of claim 48, for use in mapping regions of known sequence in a target polynucleotide which is in a substantially extended condition, wherein the target is contacted with a plurality of PREs, each having different surface-attached oligonucleotides effective to hybridize to one of the know-sequence regions of the target, said contacting is carried out under conditions which allow the PRE's surface-attached oligonucleotides to hybridize with the target's selected base sequences, and said detecting includes (i) washing the field to remove unbound PREs, and (ii) mapping the relative positions of the bound PREs according to their spectral emission characteristics.

50. The method of claim 40, for resolving the distance between two closely spaced target sites, wherein said PREs have substantially the same peak wavelength, wherein said detecting includes detecting a composite spectral emission characteristic of the two PREs including shifts and broadening of single-particle spectral peaks and appearance of new peaks.

51. The method of claim 40, for resolving the distance between two closely spaced target sites, wherein said PREs have different peak wavelengths, wherein said detecting includes separately detecting the center of the diffraction peak of each particles at different illuminating light wavelengths.

52. The method of claim 41, wherein said target includes an array of different-sequence oligo- or polynucleotides, the array is contacted with one or more PREs having one or more surface-attached polynucleotides, said contacting is carried out under conditions which allow the PRE's surface-attached polynucleotides to hybridize with the target array oligo- or polynucleotides, and said detecting includes (i) washing the target to remove unbound PREs, and (ii) detecting a spectral emission characteristic of PREs at each region of the array.

53. The method of claim 41, wherein said target is a polynucleotide present as a separated band in an electrophoresis gel, said contacting is carried out by exposing the surface of the gel to PREs under hybridization conditions.

54. The method of claim 41, wherein the molecular feature of interest is a molecule which functions catalytically to break a bond between two atoms in a molecular chain, said PRE includes a pair of PREs linked by said chain, said contacting is carried out under conditions effective to cleave the molecular chain, and said detecting includes detecting the disappearance of linked PREs or the appearance of unlinked PREs.

55. The method of claim 41, for detecting the presence of a target polynucleotide sequence having first and second contiguous nucleotide sequences, said contacting includes adding to the target, under hybridization conditions, first and second PREs having surface-localized first and second oligonucleotide probes complementary to the first and second target sequences, respectively, and treating the resulting hybridization product with a ligase enzyme, and said detecting includes detecting the presence of linked PREs.

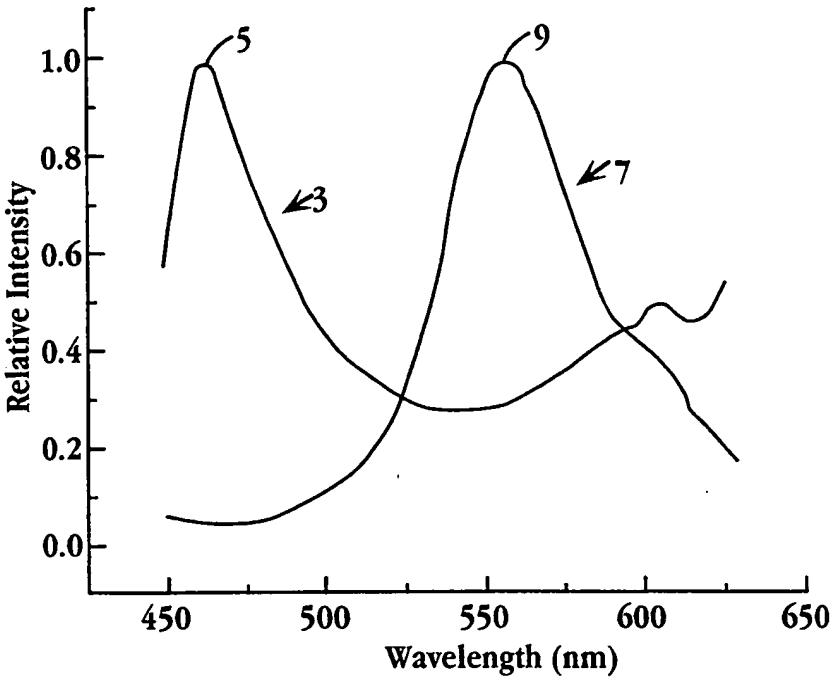

Fig. 1

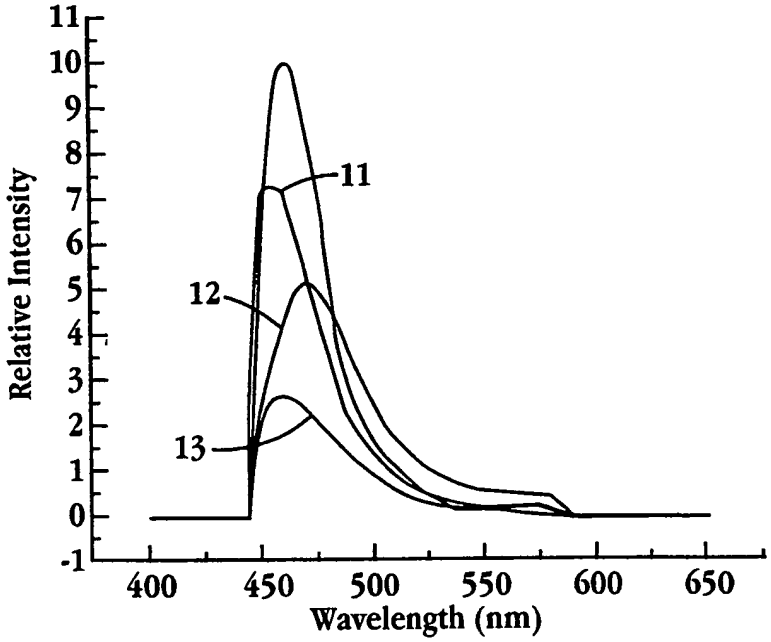

Fig. 2

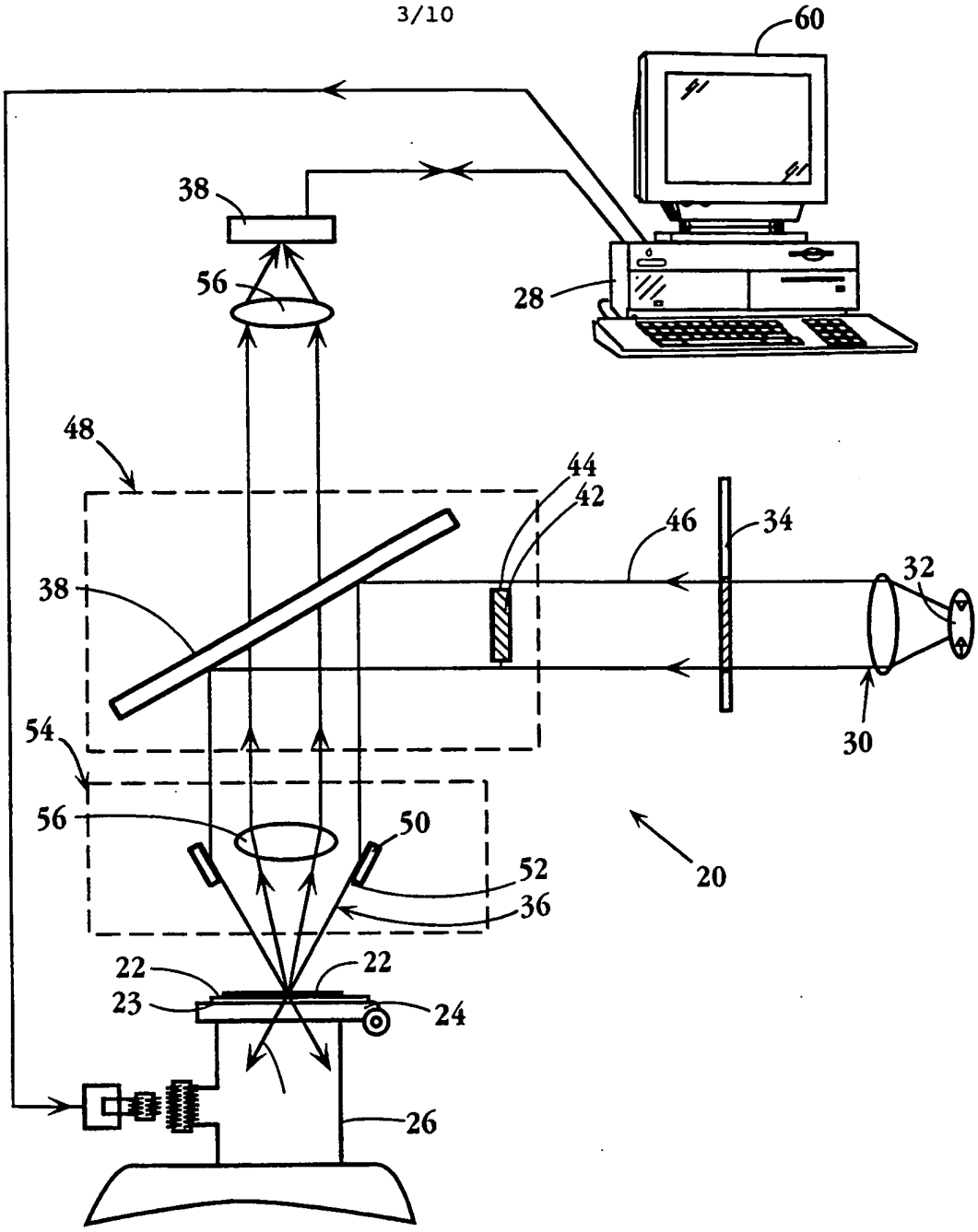

Fig. 3

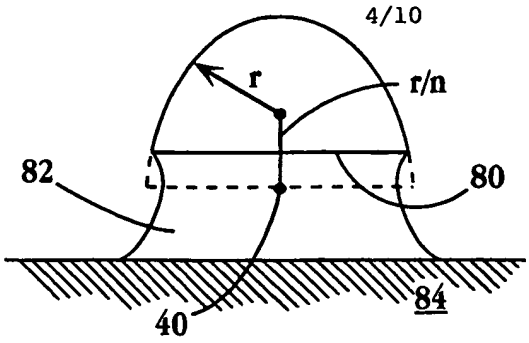

Fig. 4

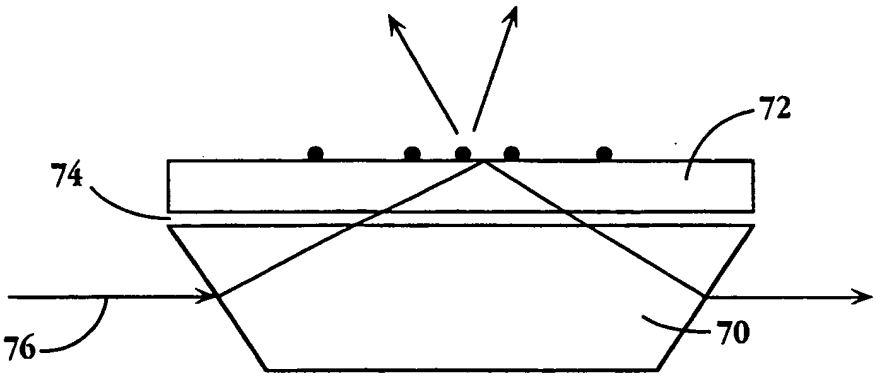

Fig. 5

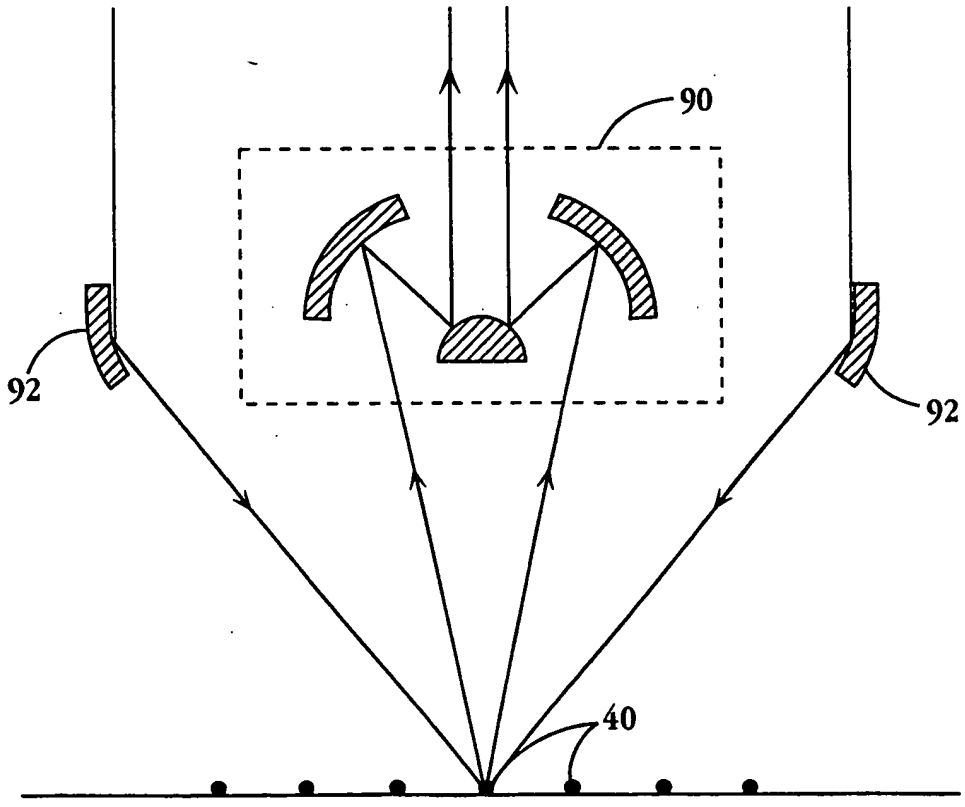

Fig. 6

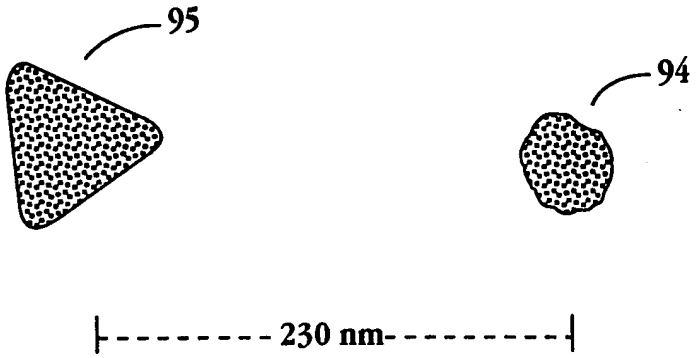

Fig. 7

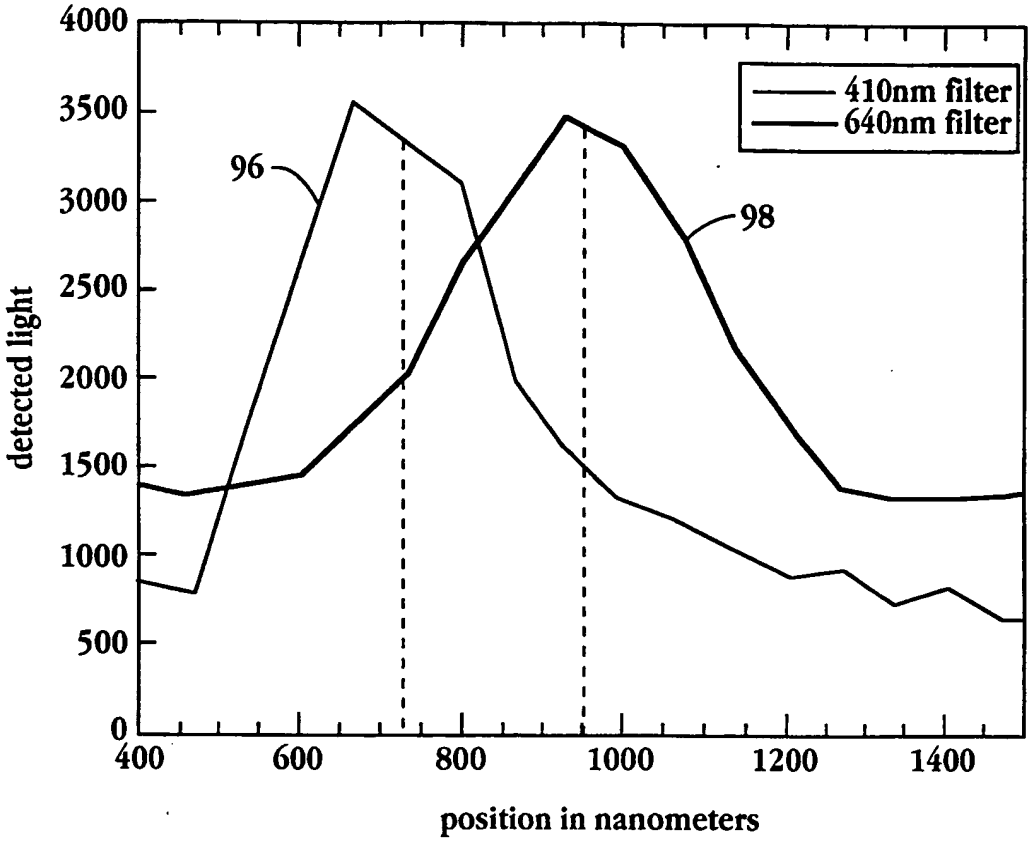

Fig. 8

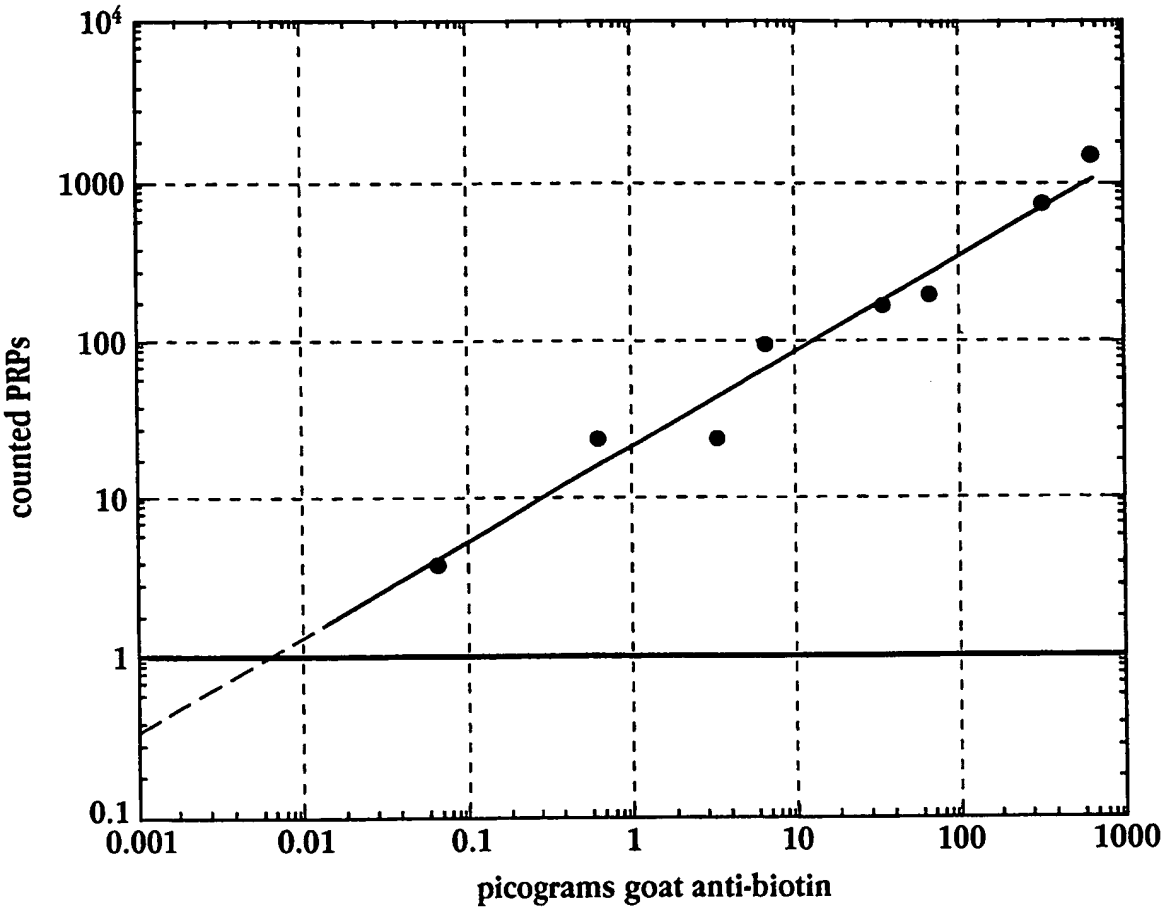

Fig. 9

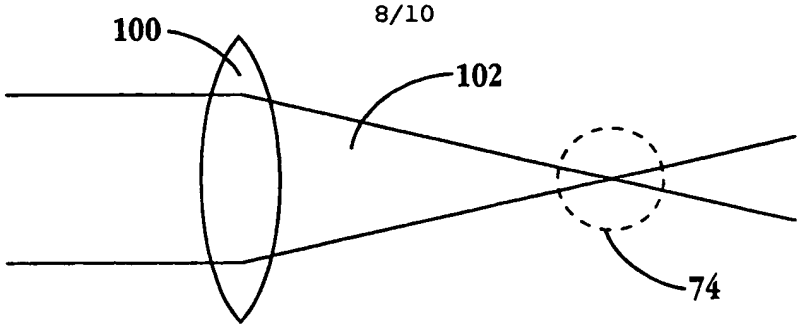

Fig. 10A

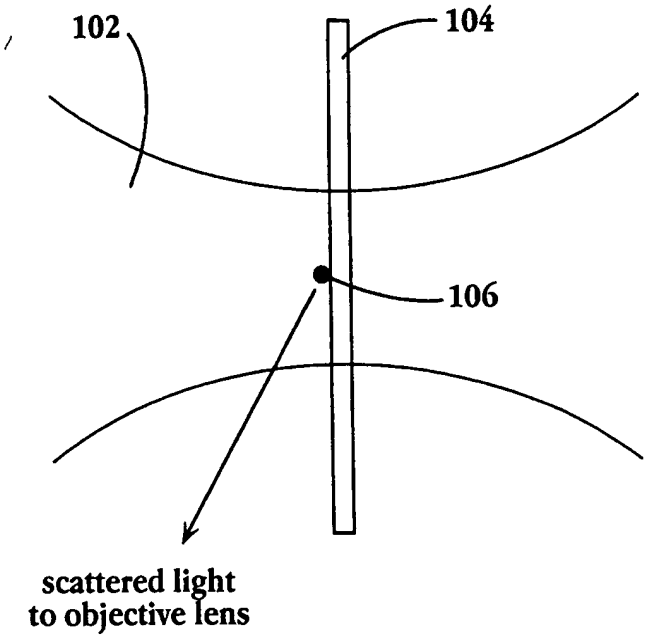

Fig. 10B

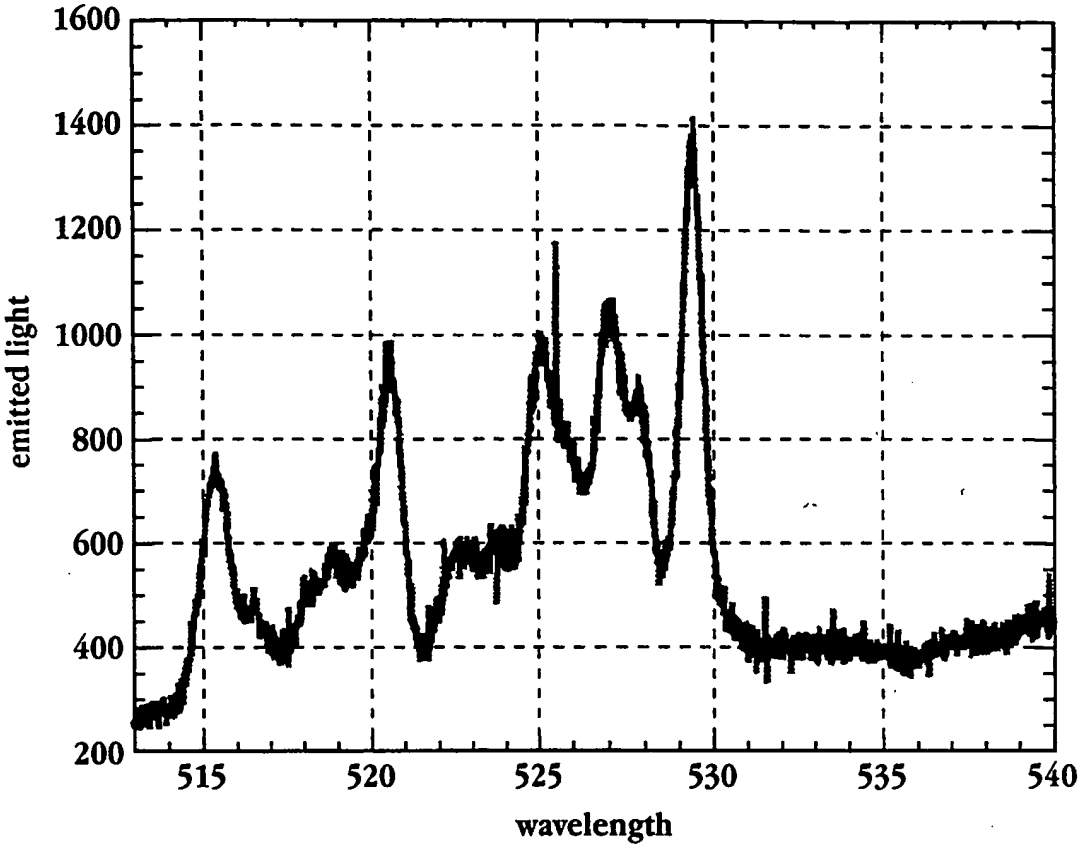

Fig. 11

Fig. 12

Fig. 13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/02995

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 33/553
US CL :436/525

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A ---- X	US 4,979,821 A (SCHUTT et al) 25 December 1990, see entire document.	1-29, 40-56 ---------- 30-39
X	US 4,313,734 A (LEUVERING) 02 February 1982, see entire document.	30-39

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"B"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 31 MAY 1998	Date of mailing of the international search report 19 JUN 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer CHRISTOPHER CHIN Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/02995

B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S.

356/300, 301, 317, 318, 337, 244, 246;
422/55, 82.05, 82.08, 82.11;
435/7.9, 176, 287.1, 287.2, 288.3, 288.7, 808;
436/169, 524, 525, 172, 805, 815